

EXHIBIT H

Improved Fluorescence and Dual Color Detection with Enhanced Blue and Green Variants of the Green Fluorescent Protein*

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The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is a versatile reporter protein for monitoring gene expression and protein localization in a variety of systems. Applications using GFP reporters have expanded greatly due to the availability of mutants with altered spectral properties, including several blue emission variants, all of which contain the single point mutation Tyr-66 to His in the chromophore region of the protein. However, previously described "BFP" reporters have limited utility, primarily due to relatively dim fluorescence and low expression levels attained in higher eukaryotes with such variants. To improve upon these qualities, we have combined a blue emission mutant of GFP containing four point mutations (Phe-64 to Leu, Ser-65 to Thr, Tyr-66 to His, and Tyr-145 to Phe) with a synthetic gene sequence containing codons preferentially found in highly expressed human proteins. These mutations were chosen to optimize expression of properly folded fluorescent protein in mammalian cells cultured at 37 °C and to maximize signal intensity. The combination of improved fluorescence and higher expression levels yield an enhanced blue fluorescent protein that provides greater sensitivity and is suitable for dual color detection with green-emitting fluorophores.

The cloning of the wild-type green fluorescent protein (GFP)¹ gene (wtGFP) (1) from the jellyfish *Aequorea victoria* and its subsequent expression in heterologous systems (2–5) has established GFP as a powerful reporter for the analysis of gene expression and protein localization in a wide variety of experimental designs. The principal advantage of GFP reporter systems is the ability to detect fluorescence in living specimens with real-time kinetics. In recent years, a number of different "red-shifted" (6–9) and UV-optimized (10, 11) GFP variants have been described that largely overcome the limitations of wtGFP for single reporter applications. These GFP variants provide an increased signal intensity, faster rate of oxidative chromophore formation, resistance to photobleaching, and ex-

citation maxima better suited to conventional detection instruments (4, 5). Despite these many improvements, one important feature shared by wtGFP and each of these variants remains the same: they all produce green light.

Various blue emission mutants of GFP have been reported previously (4, 12) but have lacked broad utility due to the relatively low fluorescence intensities produced by these reporter proteins. The initial variant of this type referred to as P4 (7) contains the single point mutation Tyr-66 to His in the chromophore region of the protein and yields a strong cobalt blue signal but only dim fluorescence. An improvement in this variant termed P4–3 (4) contains an additional Tyr-145 to Phe substitution that improves the folding properties of the protein and thereby the subsequent fluorescence output. The P4–3 double mutant is approximately 2-fold brighter than P4, primarily due to a higher fluorescence quantum yield (QY). Moreover, all previous reports with blue emission variants such as P4–3 have used GFP genes containing wild-type jellyfish codons, which leads to inefficient expression in higher eukaryotes (13).

In the present study, we describe a blue emission variant of GFP termed EBFP, which contains four point mutations: Phe-64 to Leu, Ser-65 to Thr, Tyr-66 to His, and Tyr-145 to Phe. We and others have shown previously that the Phe-64 to Leu substitution improves the efficiency of chromophore formation at 37 °C, thereby increasing the intracellular level of functional protein expressed at this temperature (14, 15). These mutations were placed in a coding sequence that was further modified with approximately 190 silent base changes to contain codons preferentially found in highly expressed human proteins (13, 16). The "humanized" backbone used in EBFP contributes to efficient expression of this variant in mammalian cells and subsequently brighter fluorescent signals. We further illustrate that EBFP can be used in conjunction with a humanized red-shifted variant termed EGFP (16) for dual color detection in mammalian cells by flow cytometry.

EXPERIMENTAL PROCEDURES

DNA Manipulations—The *A. victoria* GFP cDNA was obtained from the plasmid TU#65 (2). The EGFP cDNA (16) was obtained from Clontech Laboratories, Inc. (Palo Alto, CA) in the plasmid pEGFP-C1. The EGFP/hyg construct shown in Fig. 4 was as previously reported (17). Oligonucleotide-directed mutagenesis was used to generate the EBFP cDNA by targeting codons for amino acid positions 66 and 145 in the EGFP coding sequence with the transformer site-directed mutagenesis kit (Clontech). Mutagenesis of pEGFP-C1 yielded the construct named pEBFP-C1. Oligonucleotide-directed mutagenesis was used to generate a human codon-optimized cDNA encoding the P4–3 variant by targeting codons for amino acid positions 66 and 145 in the wtGFP coding sequence in a similar fashion. Identical mammalian expression vectors containing the cytomegalovirus immediate early promoter and SV40 polyadenylation signal were used to express the humanized form of

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¹ The abbreviations used are: GFP, green fluorescent protein; BFP, blue fluorescent protein; wtGFP, wild-type green fluorescent protein; EBFP, blue emission variant of GFP; EGFP, red-shifted emission variant of GFP; QY, quantum yield.

P4-3 and EBFP. To obtain the biophysical data shown in Table I and Fig. 1, wtGFP and EBFP cDNAs were subcloned in frame with a polyhistidine tag in the T7 expression vector pRSETA (Invitrogen Corp., San Diego, CA) to produce N-terminal 6[*mult*]His-wtGFP and 6[*mult*]His-EBFP expression constructs.

Cell Lines and Method of Culture—293 EBNA cells (Invitrogen) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 5% Pen/strep (Life Technologies, Inc.). NIH/3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose and 10% newborn calf serum. K562 cells were cultured in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, and 50 μ g/ml gentamicin sulfate. Each cell line was propagated at 37 °C in a humidified atmosphere of 95% air, 5% CO₂.

Transfection of Mammalian Cells—For 293 EBNA and NIH/3T3 cells, $1-4 \times 10^5$ cells were seeded in tissue culture plates and cultured for 18–24 h in the appropriate growth medium supplemented with serum. Transfections were performed using the LipofectAMINE® reagent (Life Technologies, Inc.) according to the instructions provided by the manufacturer. The cells were incubated in the transfection solution containing DNA/lipid for 8–12 h at 37 °C in a humidified atmosphere of 95% air, 5% CO₂. For K562 cells, 4×10^6 cells were electroporated with 30 μ g of DNA in a 250- μ l volume (complete growth medium) at 250 V using a Bio-Rad Gene Pulser II. The cells were allowed to recover for various periods in complete medium as indicated in the appropriate legend, and GFP fluorescence was detected by fluorescence microscopy or flow cytometry.

Biophysical Characterizations—6 \times His-wtGFP and 6 \times His-EBFP were expressed in *Escherichia coli* and purified by affinity chromatography on Ni²⁺-NTA columns (Qiagen, Inc., Thousand Oaks, CA). Purified protein concentrations were determined by the BCA assay, and the purification efficiencies were estimated by scanning densitometry of column fractions on SDS gels stained with Coomassie Blue. Spectra were obtained from equal concentrations of wtGFP and EBFP protein using a SPEX 1681 Fluorolog spectrofluorometer (Edison, NJ) with a 250 W xenon arc lamp. The extinction coefficient (E_M) for EBFP was calculated using Beer's law and the absorbance at 380 nm with 7 μ M protein expressed at 28 °C. The EBFP QY was determined using emission at 390–550 nm (excitation at 380 nm) in comparison to an equal optical density of 1-aminoanthracene (Aldrich; QY = 0.61) in cyclohexane as a reference standard.

Flow Cytometry—The flow cytometric analysis shown in Fig. 2 was performed using a FACStar PLUS cytometer (Becton Dickinson, Inc., San Jose, CA) equipped with a 4 W multiline visible argon-ion laser tuned to 488 nm with 300 mW power. Green fluorescence pulses were collected through a 530/30 nm bandpass filter. Blue fluorescence was excited using a 5 W argon-ion laser tuned to UV output (351–364 nm) at 200 mW power and emission detected with a 424/44 bandpass filter. Photomultiplier pulses were amplified logarithmically. The dual color detection of EBFP and EGFP shown in Fig. 4 was performed using a FACS Vantage cytometer (Becton Dickinson). EGFP was excited at 488 nm and emission detected using a 510/20 bandpass filter. EBFP was excited by UV and detected using a 424/44 bandpass filter.

Fluorescence Microscopy—Microscopy was performed using a Zeiss Axioscope model 50 fluorescent microscope. The filter set used to detect EBFP fluorescence was from Chroma Technology, Inc., (Burlington, VT, catalog number 31021). The excitation and emission wavelengths for this filter set are 390/22 nm and 460/50 nm, respectively. Photographs were taken using Kodak Ektachrome 400 ASA film using a 15-s exposure.

RESULTS

Enhanced Blue Emission Variant of GFP—A comparison of the spectral properties for three previously described blue emission variants of GFP is shown in Table I. The variants P4, P4-3, and BFP5 (18) have met with limited utility due to the dim fluorescence produced by these proteins, which is reflected in the relatively low E_M and QY values for each variant. Moreover, we and others have shown that the GFP cDNA is poorly expressed in higher eukaryotes due to inefficient use of jellyfish codons in plants and mammalian cells (13, 16). To improve upon each of these qualities, we have combined a blue emission mutant of GFP having a higher E_M value with a re-engineered GFP gene sequence containing codons preferentially found in

TABLE I
Spectral properties of different blue emission variants of GFP

Variant	Mutations	Excitation max.	Emission max.	E_M	QY
		nm	nm		
Wild type	None	395 (470)	509	21,000 (7,150)	0.77
P4	Y66H	383	447	13,500	0.21
P4-3	Y66H	381	445	14,000	0.38
	Y145F				
BFP5	F64M	385	450	ND	ND
	Y66H				
	V68I				
EBFP	F64L	380	440	31,500	~0.20
	S65T				
	Y66H				
	Y145F				

ND, not determined.

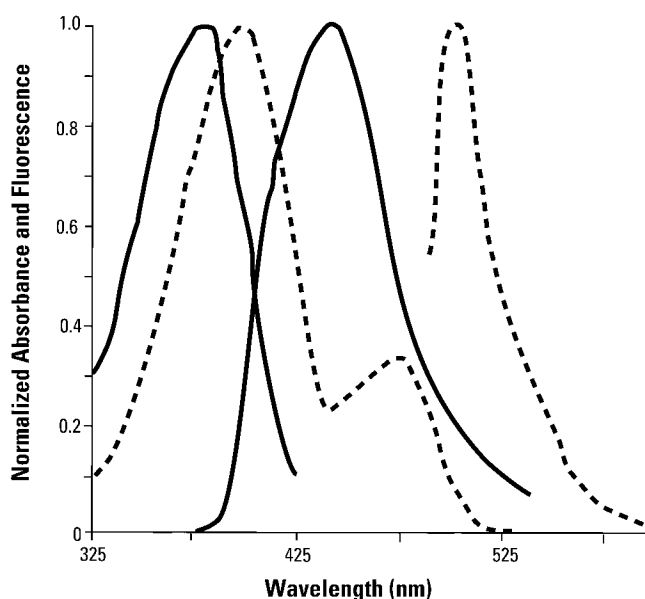


FIG. 1. Fluorescence excitation and emission spectra of recombinant EBFP (solid lines) and wtGFP protein (dashed lines). Spectra were obtained from equal concentrations of 6 \times His-tagged protein on a spectrofluorometer as described under "Experimental Procedures."

highly expressed human proteins (13). We have termed this improved variant EBFP.

As shown in Table I, the enhanced signal intensity of EBFP relative to the other blue emission variants is due in part to an increase in the E_M (31,500 cm⁻¹ M⁻¹ at 380 nm). The fluorescence QY of each BFP variant is similar, 0.38 for P4-3 being the highest, and ~0.20 for EBFP and P4. As the product of the E_M and QY is proportional to the fluorescence intensity of a given fluorophore, EBFP yields a stronger signal relative to P4-3 despite the lower QY. The EBFP protein has excitation and emission maxima of 380 and 440 nm, respectively (Table I; the complete fluorescence spectra for EBFP shown in comparison to wtGFP are shown in Fig. 1). These maxima are similar to those reported previously for other GFP variants containing the Tyr-66 to His mutation. The maxima for wtGFP were similar to values described previously (2) at 397 nm (minor peak at 475 nm) for excitation and 504 nm for emission. Recombinant expression of 6 \times His-tagged wtGFP and GFP variants have been previously shown not to alter the fluorescence spectra of these proteins (8, 19).

A comparison of the fluorescence intensities of EBFP and P4-3 expressed in mammalian cells is shown in Fig. 2, where transiently transfected 293 EBNA cells expressing each variant were quantified by flow cytometry. The fraction of cells

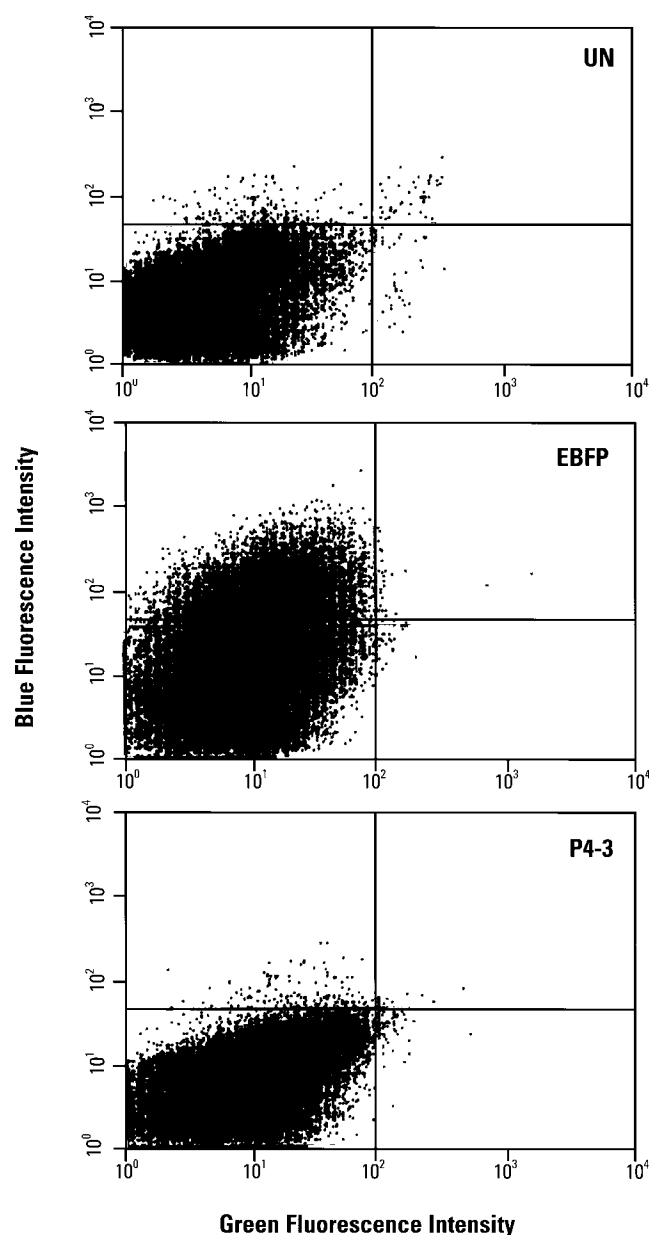


FIG. 2. **Quantitative comparison of P4-3 and EBFP blue emission variants by flow cytometry.** Identical mammalian expression vectors were used to express the humanized form of P4-3 and EBFP in 293 EBNA cells and the cells prepared for analysis 48 h post-transfection. Green fluorescence pulses were collected through a 530/30 nm bandpass filter, whereas blue fluorescence was detected using a 424/44 nm bandpass filter. The fraction of cells showing elevated blue fluorescence (monitored in the FL4 channel) for EBFP and P4-3 were each normalized to values obtained for untransfected (UN) cells.

showing elevated blue fluorescence (monitored in the FL4 channel) was 23.3% for EBFP-expressing cells and 0.35% for P4-3 (values normalized to untransfected (UN) cells). The difference in signal intensity is unlikely related to transfection or expression efficiencies, as EBFP and P4-3 were encoded by identical humanized backbones. Moreover, P4-3 has essentially the same spectral maxima as EBFP (P4-3 excitation and emission maxima of 381 and 445 nm, respectively) (4) and is thereby detected at equivalent signal-to-background levels using these filters. Based solely on differences between the respective E_M and QY values for EBFP and P4-3 there is an expected 18% difference in signal intensity. Therefore, the brighter signal for EBFP observed in transfected mammalian cells (Fig. 2) is likely due to the combination of a higher E_M

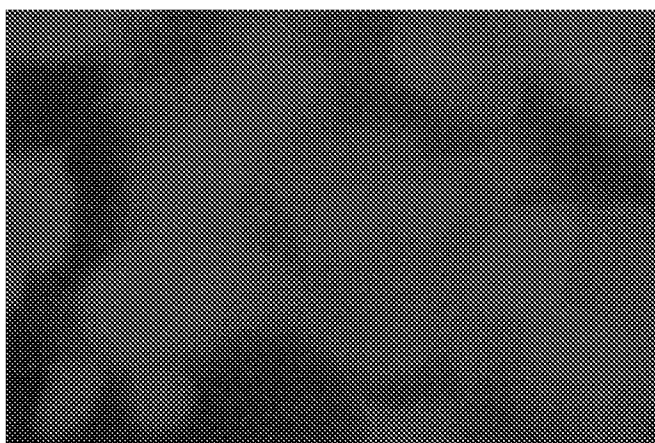


FIG. 3. **Expression of EBFP in NIH/3T3 cells and detection by fluorescence microscopy.** The expression vector pEBFP-C1 was transiently transfected into NIH/3T3 cells using a liposome transfection method. At 72 h post-transfection, the cells were visualized by fluorescence microscopy using a Zeiss Axioscope model 50 microscope fitted with a bandpass filter set having excitation and emission wavelengths of 390/22 and 460/50 nm, respectively. Photographs were taken with a 40 \times objective and a 15-s exposure.

value and a greater percentage of protein having a properly folded chromophore by virtue of the Phe-64 to Leu mutation (14). Negligible green fluorescence (FL1 channel) was detected for all three cell populations, indicating that EBFP is suitable for co-expression with markers having green emission. Last, as shown in Fig. 3, cytoplasmic expression of EBFP in NIH/3T3 cells yields a blue signal that is easily detected by conventional epifluorescence microscopy.

Dual Color Detection of EGFP and EBFP—The properties of the red-shifted and UV-optimized GFP variants largely overcome the limitations of wtGFP for single reporter studies. Combinations of GFP variants can be used for dual reporter applications by using selective excitation conditions (6, 20), but this process is complicated for microscopy, as the image collected from each reporter is green. Dual color images must be generated by pseudocoloring techniques (6, 20) or by depicting separate images for each variant. Moreover, the utility of green emitting reporters is limited in both microscopy and flow cytometry in cases in which cellular green autofluorescence is a concern or when the reporter is used in conjunction with chemical fluorophores such as fluorescein. For each of these reasons, it is desirable to have emission variants of GFP capable of producing distinct colors for use in dual color applications.

The utility of EGFP and EBFP in dual color applications was investigated by transfection of K562 cells with either pEBFP-C1 or a construct encoding a hygromycin resistance/EGFP fusion gene (EGFP/hyg) (17). Flow cytometric analysis of these cells 48 h after transfection is illustrated in Fig. 4. A and B show the fluorescence detected from K562 cells that were transfected with the EGFP/hyg or the EBFP constructs, respectively. Comparison of the EGFP and EBFP fluorescence reveals the expected difference in the signal intensity of these variants, which reflects the higher E_M and QY values for EGFP (16). The cells analyzed in C represent a 50:50 mixture of the independently transfected cells shown in A and B. These data clearly show that the fluorescence from these two GFP variants can be easily distinguished from one another and demonstrate the utility of these proteins as independent reporters that can be detected simultaneously. D shows the level of background fluorescence from mock-transfected cells, indicating that cellular autofluorescence does not interfere with the detection of each reporter. Last, E shows the results from the flow cytometric analysis of K562 cells that were co-transfected with both the

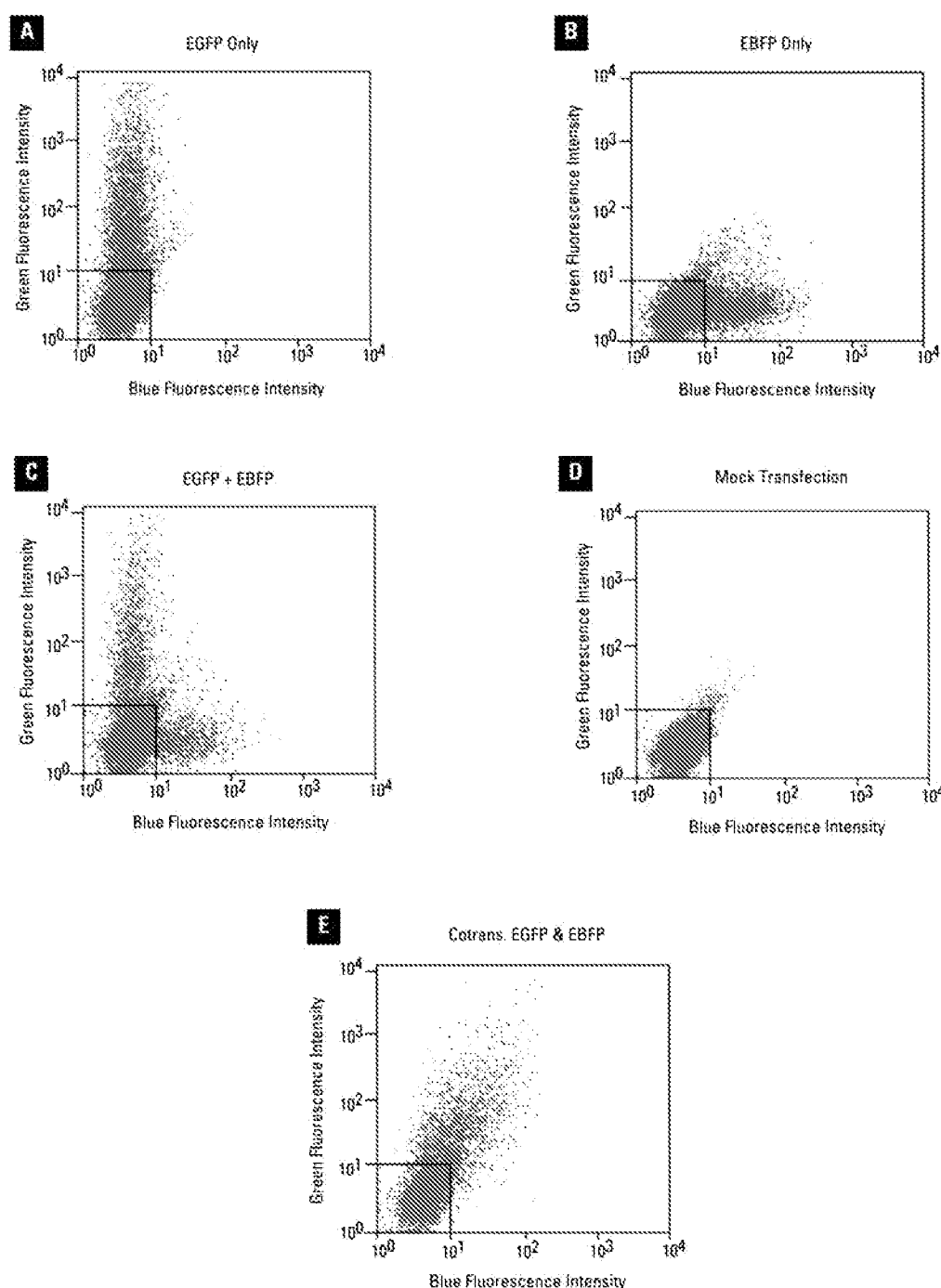


FIG. 4. **Flow cytometric analysis of EGFP- and EBFP-expressing cells.** K562 cells were transfected by electroporation and analyzed 48 h later with a FACS Vantage flow cytometer. EGFP fluorescence was excited using a 488 nm laser output and emission was detected using a 510/20 nm bandpass filter. EBFP fluorescence was stimulated using UV excitation and emission was detected using a 424/40 nm bandpass filter. *A*, analysis of cells transfected with the EGFP/hyg construct alone. *B*, cells transfected with the EBFP construct alone. *C*, analysis of a 50:50 mixture of the cells analyzed in *A* and *B*. *D*, mock-transfected cells (negative control). *E*, analysis of cells that were co-transfected with both the EGFP/hyg and EBFP constructs.

EGFP/hyg and EBFP constructs. Cells expressing both proteins are easily detected, and the pattern of fluorescence from these cells is clearly distinguishable from cells that were singly transfected (*C*). Taken together, these results demonstrate that EGFP and EBFP can be used as independent reporters whose expression can be simultaneously analyzed by flow cytometry.

DISCUSSION

In the present study, we have described a blue emission variant of GFP termed EBFP, which yields a bright fluorescent signal in mammalian cells due to the combined effects of mu-

tations that enhance the folding and spectral properties of the protein and use of a synthetic coding sequence to improve expression levels in transfected cells. This variant is more effectively excited relative to previously described BFP reporters as measured by an elevated E_M value. The efficiency of fluorescence emission remains relatively low, as the QY of EBFP is similar to previously reported values for P4 (7). Comparison of EBFP to P4-3 in transiently transfected mammalian cells indicates that the EBFP variant provides superior sensitivity as measured by flow cytometry.

There are several factors that may contribute to the enhanced signal intensity observed for EBFP. We have previously shown that a human codon-optimized sequence can enhance expression levels by ~4-fold in mammalian cells relative to equivalent GFP coding sequences containing jellyfish codons (16). However, the results shown in Fig. 2 indicate that enhanced expression levels are not responsible for the elevated blue fluorescence of EBFP relative to P4-3, as both cDNAs are "humanized" in this experiment. Neither can the larger E_M for EBFP alone account for the increase in signal intensity. Previous studies with several GFP variants have revealed that only those mutants containing the Phe-64 to Leu mutation could be expressed in a fluorescent forum at 37 °C (15). Moreover, we have shown that equal concentrations of EBFP expressed at either 28 or 37 °C have similar E_M values (14), indicating that similar folding efficiencies and chromophore formation were achieved at each temperature. Therefore, it is likely that in addition to the increased E_M value of EBFP, enhanced signal intensity from protein expressed in mammalian cells (Fig. 2) is due to an increase in the intracellular concentration of functional fluorescent protein. Recent findings indicate that the Ser-65 to Thr mutation is not as critical, as a blue emission mutant lacking this change (e.g. Phe-64 to Leu, Tyr-66 to His, and Tyr-145 to Phe) yields signals comparable to EBFP (data not shown). As most mammalian cell lines require culture temperatures near 37 °C, the effect of the Phe-64 to Leu mutation points to a practical advantage of EBFP relative to other blue emission mutants of GFP.

For single reporter applications in mammalian cells codon-optimized red-shifted variants such as EGFP fulfill most needs. EGFP has properties that overcome limitations associated with wtGFP, including increased fluorescence intensity when excited by blue light (488 nm), a shorter lag in the development of fluorescence after protein synthesis, and improved expression in most cell types (9, 16). Therefore it appears that the main application for blue emission variants such as EBFP is for use as a second fluorescent reporter in combination with GFPs and other green fluorophores. In this regard, EBFP/EGFP combinations should prove useful to monitor the activity of two different promoter elements or to detect the localization of two different proteins inside the same cell, tissue, or organism. This latter approach has been demonstrated using fusion constructs encoding a P4-3 derivative targeted to mitochondria together with a nuclear targeted GFP to monitor each organelle simultaneously in living HeLa cells (4). The data shown in Fig. 4 of the present study illustrate that EGFP and EBFP can be distinguished in a mixed population of cells or when co-expressed in the same cells. This technique may extend previous flow cytometric studies for characterizing GFP vectors used in *in vivo* gene delivery (21-23) or in transgenic studies (24) with reporters targeted to different tissue or cell types.

As the list of GFP variants continues to grow, it is likely that applications using these new reporter proteins will also expand at a rapid pace. The combination of EGFP and EBFP with the recently described clone 10C variant (25) that yields yellow-green fluorescence (excitation maxima 513 nm and emission maxima 527 nm) makes it feasible with the appropriate filter sets to conduct three-color detection by either flow cytometry or fluorescence microscopy (4). Multiparameter detection of distinct spectral variants opens up a wide range of applications such as the simultaneous analysis of multiple gene expression patterns, intracellular localization of several different proteins, real-time analysis of protein-protein interactions utilizing fluorescence resonance energy transfer (4), and monitoring the lineage of different cell populations.

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